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Lymphoid Potential, Probed before Circulation in Mouse, Is Restricted to Caudal Intraembryonic Splanchnopleura

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Summary

Emergence of hemopoietic stem cells in the mammalian embryo has yet to be definitively allocated. Previously, we detected multipotent hemopoietic precursors in the region surrounding the dorsal aorta (paraaortic splanchnopleura) beginning at 8.5 days postcoitum (dpc). However, as circulation is already established, it remained unclear whether hemopoietic precursors arise in situ or are blood-delivered. By adding an organotypic step to our former culture system, we now detect lymphocyte and multipotent myeloid precursors from the intraembryonic splanchnopleura as early as 7.5 dpc. Under identical conditions, yolk sacs from the same embryos are unable to generate lymphoid progeny and have a reduced potential for myeloid differentiation and maintenance. Thus, if isolated before circulation, the yolk sac does not produce multipotent precursors and therefore does not contribute to definitive hemopoiesis in the mouse.

Introduction

The embryonic environment is thought to provide specific conditions for the emergence and expansion of hemopoietic stem cells (HSC), which are not encountered in adult bone marrow. It is crucial to investigate further the initial steps of hemopoietic development in order to gain insight concerning the control of determination and renewal of HSC. The yolk sac (YS) was previously believed to be the unique provider of colonizing HSC in the embryo as no other embryonic tissue was found to have hemopoietic potential (Moore and Metcalf, 1970). Attention was drawn to sites within the embryo proper, following the demonstration in the avian model (Dieterlen-Lièvre, 1975) that definitive hemopoiesis was initiated by intraembryonic precursors. Studies in the mouse focused on the time preceding fetal liver (FL) colonization by HSC, which occurs at 10 days postcoitum (dpc), at the 28–32 somite stage. The first evidence of intraembryonic hemopoietic potential was obtained by cultivating cells from whole embryos severed from their YS, in conditions that promoted differentiation along the myeloid (Huang et al., 1994) or B-lymphoid (Ogawa et al., 1988; Cumano et al., 1993; Huang et al., 1994) lineages. These authors established that cells displaying such potential were present in the embryo around day 9 of gestation, without, however, determining the structures involved.

Since the region giving rise to definitive HSC neighbors the dorsal aorta in birds, we looked for and found cells with hemopoietic potential in the corresponding region of the mouse embryo (Godin et al., 1993, 1995a, 1995b). This region included splanchnic mesoderm surrounding the endoderm of the developing gut and the endothelium of arteries. We named it paraaortic splanchnopleura (P-Sp), as splanchnopleura (Sp) designates associated endodermal and mesodermal germ layers of vertebrate embryos (Hamilton, 1952). In these studies, in vitro analysis of dissociated cells from the P-Sp or from the embryonic body without P-Sp demonstrated that intraembryonic precursors were exclusively located in this site. When YS and P-Sp were analyzed at the 10–12 somite stage, precursors appeared in both, with their number increasing in parallel reaching 15 precursors at 9.5 dpc (25 somites), which is shortly before the beginning of fetal liver colonization. Analysis of their hemopoietic potential at a single cell level revealed that the precursors in both sites were multipotent (Godin et al., 1995a). By the time of fetal liver colonization, HSC capable of long-term reconstitution of irradiated recipient mice could be detected in the AGM, comprising the aorta, gonads, and mesonephros (Müller et al., 1994).

As the blood connection between the YS and the embryo becomes established at the 8-somite stage, precursors can pass through the circulation from one site to the other. No conclusion can therefore be drawn about the origin of intraembryonic precursors in the P-Sp or AGM (aorta-gonad-mesonephros).

We thus devised a culture system whereby the splanchnopleura was allowed to grow in toto for two days after dissection from the other embryonic structures. The tissues were isolated before the establishment of circulation. We show here that, by virtue of this organ culture step, hemopoietic intraembryonic precursors can be detected in the splanchnopleura severed from the YS as early as the presomite stages (7.5–8 dpc); these precursors are thus generated in situ. The comparison between precursors from the Sp and YS obtained from the same embryo reveals that those from the splanchnopleura display a wider differentiation potential, as well as a better ability to persist in culture. Strikingly, the YS was unable to give rise to a lymphoid progeny. This finding concurs with the classical observation that lymphopoiesis does not occur in this site (Metcalf and Moore, 1971). YS cells also lacked precursors for mixed myeloid colonies (CFC-mix). This limitation of YS differentiation and renewal potential was intrinsic to YS progenitors, since the addition of Sp cells could not modify the outcome of YS cell cultures. However, shortly after the circulation between intra- and extraembryonic compartments is established, lymphoid potential becomes detectable in the YS. This result argues in favor of a seeding of the YS by Sp precursors.

As reported previously (Medvinsky et al., 1993; Huang

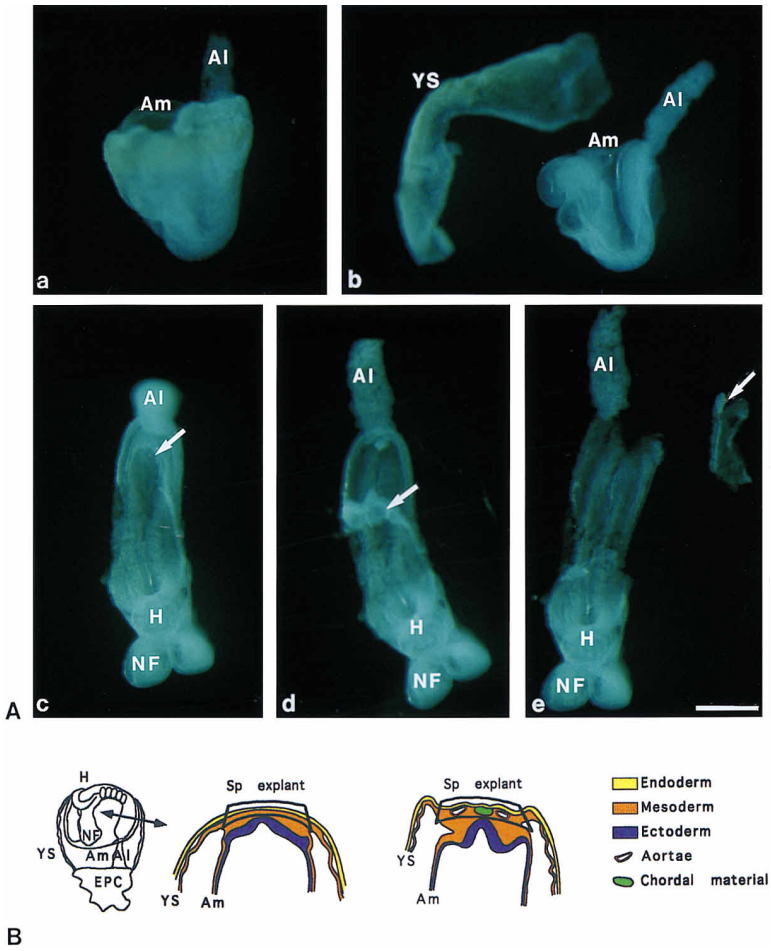

Figure 1. Dissection of YS and Sp

(A) Steps of the procedure in a 5 somite embryo. (a) Embryo still enclosed in its YS; (b) removal of the YS; (c) ventral view of the embryo after the amnion has been slit; (d) Sp half dissected out; (e) embryo after complete dissection of Sp. Al, allantoid; Am, amnion; H, heart; NF, neural folds; YS, yolk sac; arrows, splanchnopleura; bar, 0.5 mm.

(B) Schematic cross-section of the caudal part of early somite embryos. Sections were from the level indicated by the arrow on the whole embryo. (middle diagram) 2–4 somite embryo; (right diagram) 5–7 somite embryo. Al, allantoid; Am, amnion; EPC, ectoplacental cone; H, heart; NF, neural folds; YS, yolk sac.

et al., 1994; Müller et al., 1994), these pre-10.5 dpc embryonic hemopoietic progenitors are not capable of restoring the hemopoietic system of adult irradiated recipients.

Results

Hemopoietic Precursors Appear in the Intraembryonic Splanchnopleura Isolated before the Onset of Circulation

Embryos at 7.5–8.5 dpc were dissected into three fragments (Figure 1A). After resection of the YS, the associated endodermal and mesodermal layer were pulled away from the caudal part of the embryo, leaving behind the somatopleura (ectoderm and associated mesoderm). The associated endodermal and mesodermal germ layers, termed splanchnopleura, were recovered from the primitive streak region up to the somite level and lateral to the last somites (Figure 1A). The analysis of semithin sections of the caudal region of embryos indicates that the explanted region comprises undifferentiated endoderm and mesoderm before the 5-somite stage. It also contains the developing dorsal aorta and part of chordal material after this stage (Figure 1B).

The YS, Sp, and in some cases, the remainder of the embryonic body of each embryo were individually cultivated in parallel under the same conditions (Figure 2). After two days of in toto cultivation, the explants were dissociated and cells allowed to expand for three days. The erythroid-myeloid and lymphoid potential of each fragment was then individually assayed. Table 1 displays the results obtained from 46 embryos ranging from presomite stages (early allantoic bud to late head fold stage according to the criteria of Downs and Davies, 1993) to late 8.5 dpc embryos. When the three-dimensional structure was maintained in culture for two days, hemopoietic progenitors could be detected in the Sp as early as the presomite stages.

The embryonic body deprived of the YS and Sp never gave rise to hemopoietic colonies (data not shown), despite the presence of splanchnopleura in its more cephalic half. This result indicates that the splanchnopleura is regionalized regarding its potential to generate hemopoietic cell.

Splanchnopleura, but Not Yolk Sac, Generates a Lymphoid Progeny

At all stages studied, explants from both YS and Sp were able to generate myeloid cells. On the other hand, only the Sp was consistently able to give rise to lymphoid progeny. The fluorescence-activated cell sorter (FACS) profile of cells from the Sp and YS of the same 5-somite embryo, grown in B-lymphoid conditions (Figure 3A),

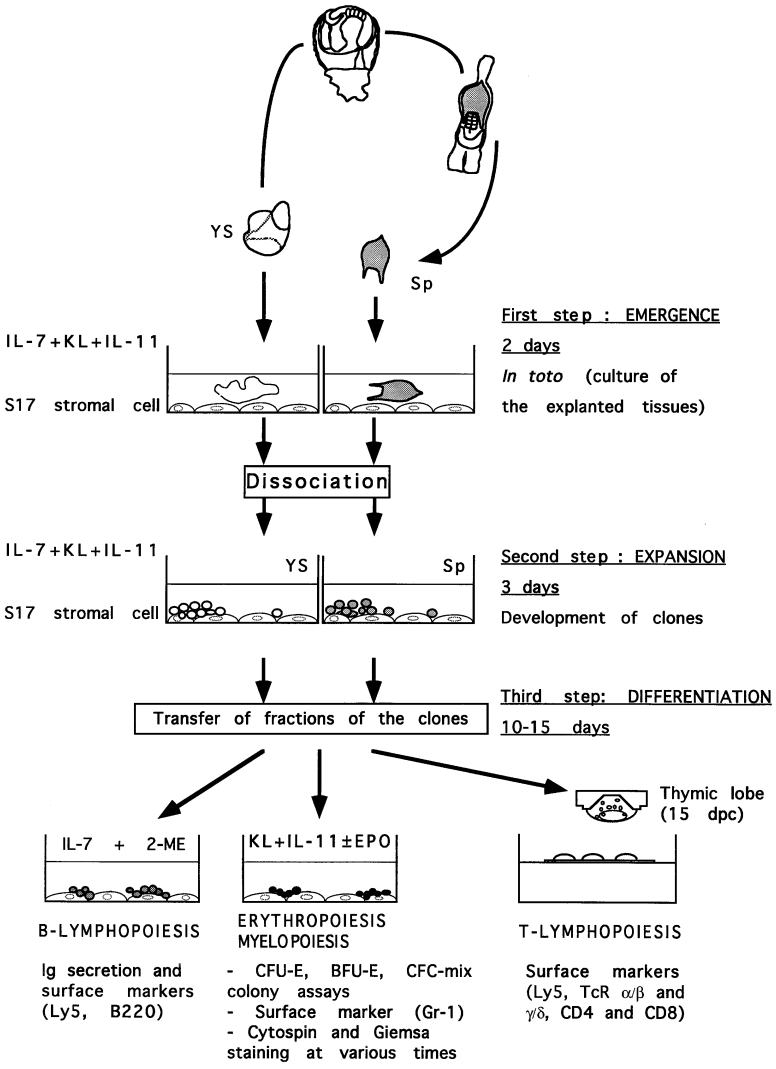

Figure 2. Three-Step Culture Assay

Scheme of the in vitro culture conditions used to detect myeloid, erythroid, B-, and T-lymphoid potentialities from early somite mouse embryos.

clearly indicates the absence of B220 positive cells in the YS culture. In contrast, these cells were already present in Sp cultures after 10 days of total culture, their number increasing conspicuously over the next 5 days to reach nearly 50% of the total cell number. When stimulated with LPS, the Sp-derived cells were capable

Table 1. Frequency of Generation of Myeloid and Lymphoid Cells by the Yolk Sac and the Sp Explanted at Various Stages

	Sp		Yolk Sac	
Stage (in Somites)	Myeloid Cells	Lymphoid Cells	Myeloid Cells	Lymphoid Cells
0	4/4	1/4	4/4	0/4
2	2/2	2/2	2/2	0/2
3	5/5	2/5	5/5	0/5
4	5/5	2/5	5/5	1/5
5	5/5	3/5	5/5	0/5
6	4/4	4/4	4/4	0/4
7	5/5	5/5	5/5	0/5
8	3/3	2/3	3/3	0/3
	Circulation			
9	6/6	5/6	6/6	1/6
10	2/2	2/2	2/2	0/2
11	3/3	3/3	3/3	0/3
15	2/2	2/2	2/2	2/2

The YS and Sp of individual embryos were tested in parallel for their ability to give rise to myeloid and lymphoid progeny as described in Figure 2. Two to six embryos were analyzed for each stage.

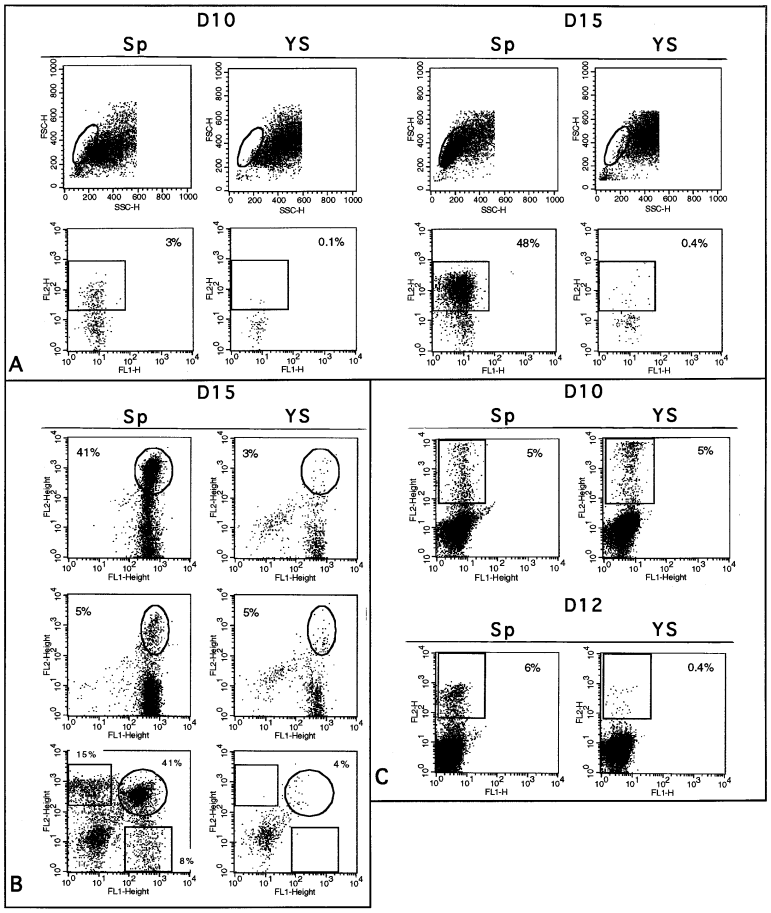

Figure 3. Phenotype of YS and Sp Cells after the Differentiation Culture Step

(A) B-lymphoid conditions. FACS profiles of YS and Sp progeny after culture in B-lymphoid conditions for 10 days and 15 days. Upper row: profiles in forward/side scatter. Lower row: cells stained with anti-B220-PE/anti-μ-FITC are displayed after gating for FSC/SSC.

(B) T lymphoid conditions. FACS profiles of cell suspensions prepared from thymic lobes 15 days after repopulation by cells grown from YS and Sp. Profiles are shown after gating for FSC/SSC. All T cells are donor-derived as identified by the anti-Ly5.1 antibody. Upper row: anti-TcR$\alpha\beta$-PE + anti-Ly5.1-FITC. Middle row: anti-TcR$\gamma\delta$-PE + anti-Ly5.1-FITC. Lower row: anti-CD4-PE + anti CD8-FITC.

(C) Myeloid conditions. FACS profile of YS and Sp progeny after culture in myeloid conditions for 10 and 13 days. Cells were stained with granulocyte-specific Gr-1-PE.

of immunoglobulin secretion, thus demonstrating that precursors from this very early embryonic site were able to fully differentiate into mature B-cells. LPS-stimulation of yolk sac-derived cells never led to immunoglobulin detection in the culture supernatant.

To test the T-lymphoid potential of the precursors generated by the YS and Sp of C57BL/6 (Ly5.1) origin, precursors were allowed to reconstitute thymic lobes from 15-dpc C57BL/6 (Ly5.2) embryos. Single cell suspensions were prepared from these repopulated thymic lobes after 10 or 15 days in culture. The expression of the donor Ly5.1 marker as well as various T-lymphocyte markers were FACS-analyzed by double staining. The cytofluorometric profile of reconstituted thymic lobes (Figure 3B) demonstrates that the potential to generate mature T lymphocytes is restricted to the Sp precursors. TcR $\alpha\beta^+$ and $\gamma\delta^+$ cells of donor origin were present only in cultures derived from Sp precursors. In addition, 50–100 fold less cells were recovered from the thymic lobes reconstituted with YS precursors, so that no more than 500–1000 events per staining could be analyzed. Moreover the four subsets of T-lymphocytes found in adult thymus, namely double negative ($CD4^-CD8^-$), single positive ($CD4^+CD8^-$ and $CD4^-CD8^+$), and double positive ($CD4^+CD8^+$), were detected in lobes colonized by Sp cells in proportions usually found in repopulated fetal thymic organ culture (FTOC), whereas these thymocytes were totally absent from YS repopulated fetal thymuses.

Yolk Sac and Splanchnopleural Environments Do Not Influence Their Respective Differentiation Potential

A possible explanation for the absence of lymphoid cells in cultures derived from early YS could be the lack of a factor favoring lymphoid differentiation. Alternatively, the YS environment might contain a factor inhibiting differentiation along the lymphoid pathway.

These possibilities were tested by analyzing the lymphoid potential of mixed YS and Sp cells. YS and Sp from Ly5.1 and Ly5.2 embryos were individually cultivated in toto for two days. After dissociation, cells from one YS and one Sp from embryos with the same somite number, but bearing different Ly5 alleles, were mixed together and further cultivated as described before. After a 12-day culture on S17 cells in the presence of IL-7, IL-11, and KL, the cells grown from the mixed populations were double-stained with B220 and Ly5.1 or Ly5.2 antibodies and analyzed by FACS. B220 positive cells derived from these cultures only bore the Ly5 allele present on the Sp cells contributing to the mixed population (data not shown).

Cell Expansion Is Exponential in Splanchnopleura and Linear in Yolk Sac Cultures

Cells from YS and Sp were expanded as described before. After the initial 2 days of in toto culture, half the cultures were supplemented with 4 U/ml of human recombinant Epo. Cells were collected at different time

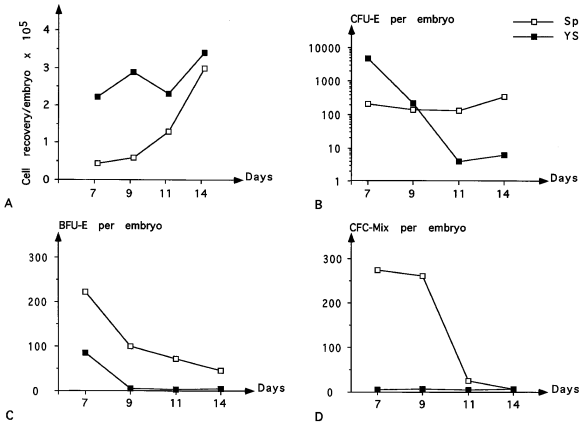

Figure 4. Quantification of Precursors in Sp and YS Cultures

Sp (n = 10) and YS (n = 10) were separated from embryos with 4 to 6 somites and submitted to the three-step culture method. Cells were retrieved at 7–14 days of step 3 and assayed for colony formation. Evolution of cell numbers (A) recovered per embryo. Evolution of CFU-E (B), BFU-E (C), and CFC-mix (D) production by Sp and YS precursors.

points starting from culture day 5. These samples were analyzed for cytology (Giemsa staining), cell surface phenotype, gene expression and for the potential to generate colonies in methylcellulose. The evolution of the numbers of cells recovered from cultures starting from day 7 until day 19 is shown in Figure 4A. In Sp-derived cultures, the number of recovered cells increased exponentially for at least two weeks, whereas no significant increase in cell numbers was found in YS cultures.

Multiple Myeloid Colonies Are Undetectable in Yolk Sac Cultures

The number of erythroid and mixed myeloid colonies generated by YS and Sp precursors at different time points is shown in Figures 4B–4D. Between days 6 and 8, the YS cells contained large numbers of late erythroid precursors (CFU-E) that rapidly disappeared. Early erythroid precursors (BFU-E) and multiple myeloid precursors (CFC-mix) were, respectively, few or absent. In contrast, CFU-E persisted in Sp cultures during the whole sampling period while BFU-E and CFC-mix were present in large numbers until day 10 of culture. This observation was consistent whether or not Epo was added during the second step of culture.

Giemsa staining of the same samples showed limited diversity of myeloid components, as well as reduced numbers of immature precursors (myeloblasts) in YS cultures as compared with Sp cultures (data not shown). Complementary cytometric analysis of YS- and Sp-derived cells for the expression of the granulocyte-specific antigen Gr-1 (Figure 3C) demonstrated that YS cultures could not sustain the production of cells from the granulocytic lineage. Gr-1 expressing cells increased in numbers in the corresponding Sp samples.

Yolk Sac Cells Do Not Express Lymphocyte-Specific Genes

YS- and Sp-derived cells were cultured as previously described, in medium supplemented with Epo during the first 13 days. mRNA was analyzed after 10, 13, and 19 days.

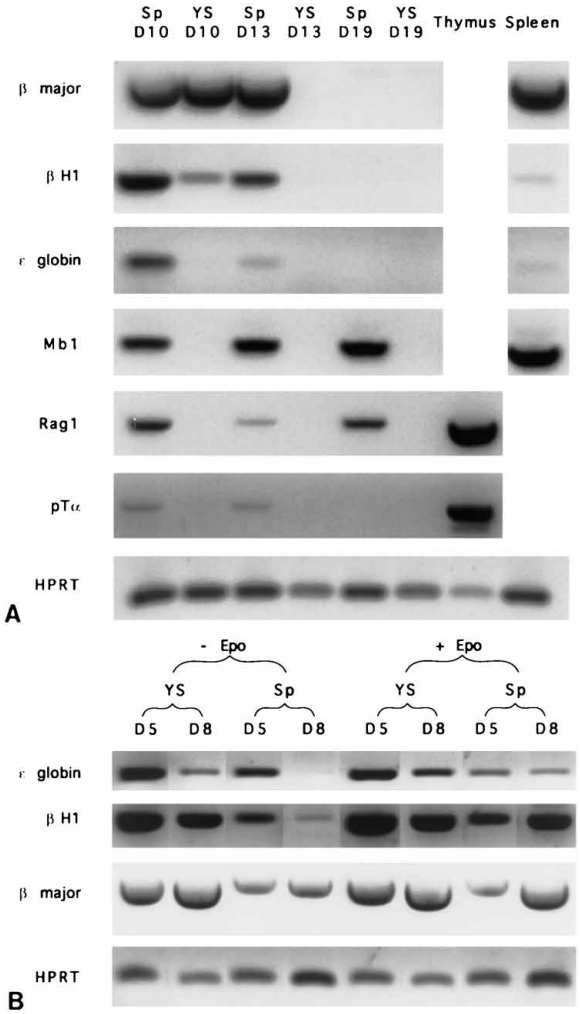

Figure 5. Expression of Genes Involved in Erythroid and Lymphoid Pathways in Cultures Derived from YS and Sp

(A) mRNA was extracted at day 10, 13, and 19 from cells recovered after the second culture step. Adult thymus and spleen were used as positive controls.

(B) mRNA was extracted at days 5 and 8 (3 and 5 days after the organotypic culture step) from YS- and Sp-derived cells cultured with or without Epo.

We monitored the expression of genes responsible for embryonic (ε and βH1) or adult (β major) globin chain production. We also investigated the expression of genes involved in lymphoid differentiation, such as RAG-1, one of the recombination activating genes (Oettinger et al., 1990); pTα, which encodes for a surrogate α chain of the T-cell receptor (Saint-Ruf et al., 1994); and mb-1, a B-cell specific Ig associated molecule (Hombach et al., 1988).

As shown in Figure 5A, the pattern of gene expression in cultured YS and Sp cells closely correlates with the morphological assessment of cell types present. The absence of a lymphoid progeny in YS cultures was confirmed by the lack of RAG-1, pTα, and mb-1 expression

at all stages studied and even under saturating polymerase chain reaction (PCR) conditions. The absence of pTα in the last sample reflects the dependence of T cell precursors on the thymic environment to complete T cell maturation.

All three β globin genes were expressed in YS and Sp samples. The rapid disappearance of erythroid gene expression in YS samples is consistent with the sharp reduction of red cells in late YS samples observed using morphological criteria. βH1 and β major globin gene expression persisted in day-13 Sp and decreased only after removal of Epo from the culture medium (Figure 5A).

In another set of experiments, the expression of globin genes was analyzed 5 and 8 days after culture. As shown in Figure 5B, irrespective of the presence of Epo, the three globin genes were expressed from the beginning of the sampling in both the YS and Sp. The decrease in ε and βH1 globin gene expression started in YS cells at day 8, correlating with the decrease observed after 10 days in culture (Figure 5A). A similar evolution was also observed in Sp-derived cells cultured in the absence of Epo, whereas, when this factor was present, increasing expression of β major and βH1 was observed.

In Vivo Reconstitution by Yolk Sac– and Splanchnopleura–Derived Cells

Cells from 3–8 somite YS and Sp were tested for their ability to reconstitute irradiated adult mice. We injected YS and Sp cells grown in vitro for 5 days on S17 feeder cells (end of the second culture step) into lethally irradiated, Ly5 different, C57BL/6 mice. After 4 to 5 months, 10 mice were analyzed for long-term reconstitution. Hemopoietic cells bearing the donor Ly5 marker were not found. We therefore tested the capacity of these cells to give rise to CFU-S (colony forming unit–spleen) d8 and CFU-S d11 after injection into the retroorbital sinus and again no donor cells were found. In an attempt to overcome a possible loss of embryonic cells at the injection site, we performed direct intrasplenic injections. The spleen of the animal injected with YS or Sp cells did not contain colonies whereas similarly injected adult bone marrow cells gave rise to CFU-S, indicating that the injection procedure was efficient.

To ascertain whether the lack of reconstitution was due to alteration of the hemopoietic precursors induced in the in vitro culture, we tested, as a control, precursors from AGM, a site whose reconstitution potential has previously been demonstrated (Medvinsky et al., 1993; Müller et al., 1994). AGM or YS from Ly5.2 embryos (35–45 somites) were dissected, dissociated, and injected in the spleen of irradiated Ly5.1 mice. After 8 days, 1–2 colonies per injected AGM and 1 colony per YS were found in the spleen. All colonies bore the Ly5.2 marker. More importantly, when YS and AGM (30–40 somites) were cultured as described before, prior to intrasplenic injection, CFU-S d8 of donor origin were found in all 12 injected mice, at a frequency of 4–5 per AGM and 2–3 per YS. These results indicate that our culture conditions did not alter the reconstitution potential of the precursors present in AGM.

Together, results from these experiments suggest that hemopoietic cells from precirculation embryos are unable to carry out hemopoiesis when transferred into an adult environment, in agreement with previous reports on 9–11 dpc embryonic cells (reviewed in Medvinski et al., 1996).

Discussion

During the last few years, much attention has been devoted to the analysis of hemopoiesis during the stage preceeding the colonization of the fetal liver by HSC. In addition to the YS, which was previously considered to be the source of colonizing cells (Moore and Metcalf, 1970), two intraembryonic sites were identified in which multipotent hemopoietic precursors are present. These were the paraaortic splanchnopleura (Godin et al., 1993, 1995a, 1995b) and the AGM, which is active shortly before and during FL colonization. The latter harbors CFU-S (Medvinsky et al., 1993, 1996) as well as cells capable of multipotent long-term reconstitution (Müller et al., 1994). Cytological signs of ongoing hemopoiesis inside the embryo could be detected at the time when the AGM acts as the intraembryonic provider of hemopoietic cells (Garcia-Porrero et al., 1995). It should be pointed out that the rudiments of all the organs constituting the AGM develop from the Sp. Whether the progenitors present in the Sp and then in the AGM are related or represent independent generations remains to be determined. Under the culture conditions employed in our previous work, intraembryonic precursors were undetectable before the blood circulates between the extra- and intraembryonic compartments. This negative outcome may result from the disruption of cell interactions necessary for the determination of precursors. Adding an organotypic culture step allowed the emergence of intraembryonic hemopoietic cells in the embryonic compartment isolated as early as the head fold stage. Working on pregastrulation stages, Kanatsu and Nishikawa (1996) recently emphasized that cell interactions are needed for hemopoietic cells to emerge.

The origin of intraembryonic hemopoietic precursors in the mouse can now be ascribed to the caudal half of the splanchnopleura. The fact that the hemopoietic potential of the splanchnopleura is restricted to its caudal region extends to earlier stages the finding previously reported in older embryos: in vivo reconstitution experiments (Tyan and Herzenberg, 1968) and in vitro studies (Cumano et al., 1993; Godin et al., 1995a) indicated that only the caudal part of the 9 dpc embryo produced hemopoietic precursors. As the YS and Sp are not yet connected by blood vessels at the stages analyzed here, the precursors in both sites represent two independent cohorts. Fate mapping studies of mouse gastrulation indicate that cells that will give rise to extraembryonic mesoderm of the YS ingress through the primitive streak earlier than their intraembryonic counterpart (Lawson et al., 1991; Parameswaran and Tam, 1995).

The two populations of precursors thus established during gastrulation can be distinguished in our study by the extent of their differentiation potential as well as by their capacity to persist in culture. In both assays, the

YS precursors at the stage studied display a reduced potential compared with those generated in the Sp. Besides the restriction of BFU-E and CFC-mix generation to the Sp precursors, the most striking result is the lack of a lymphoid progeny from YS cells. We report that mixing cells from YS and Sp did not allow the generation of lymphoid cells of YS origin. Consequently, at the stage studied, the limitation of YS cells is intrinsic and possibly results from environmental influences acting earlier at precursor emergence.

The lymphoid potential in the YS only appears following the onset of blood circulation between the YS and the embryo body. Liu and Auerbach (1991) also reported the presence of T-lymphoid precursors soon after the onset of circulation. It is tempting to conclude that precursors migrating from the splanchnopleura to the YS via the bloodstream are responsible for the newly acquired lymphoid potential. Such a translocation of intraembryonic precursors to the yolk sac was previously demonstrated in avian chimeras (Beaupain et al., 1979). Thus, the lymphoid potential detected in the YS at later stages (Weissman et al., 1978; Paige et al., 1979; Ogawa et al., 1988; Liu and Auerbach, 1991; Cumano et al., 1993; Huang et al., 1994; Godin et al., 1995a) would originate from Sp precursors having settled in the extraembryonic compartment via the blood stream. Given the lack of BFU-E and CFC-mix precursors in early YS, we suggest that intraembryonic precursors largely contribute to further erythro-myelopoiesis.

The gene expression pattern of cells derived from YS and Sp confirms the cytological and culture analyses. Transcripts exclusively expressed in lymphoid cells were never detected in YS-derived progeny. In contrast, the Sp-derived cells expressed RAG-1 as well as B (mb-1) and T (pTα) cell specific genes from early stages of culture onward. The results concur with the FACS analysis data showing the presence of B220$^+$ cells at culture day 10. The presence of transcripts for the pTα is also consistent with the potential to differentiate into T lymphocytes upon transfer into a thymic environment (FTOC). The ability of erythroid cells from both sites to express genes for embryonic (ϵ and βH1) as well as adult (β major) β-like globins extends to the gene expression level previous observations at the protein level. Adult hemoglobin is synthesized by nucleated erythrocytes from the YS, either freshly explanted (Brotherton et al., 1979) or cultured in vitro (Wong et al., 1986). Such coexpression was also observed for the α-globin chains at the mRNA level (Leder et al., 1992). YS- and Sp-derived cells differ in the evolution of globin gene expression. All transcripts studied increase in Sp samples cultured in the presence of Epo. In contrast, in YS samples these transcripts decrease with time.

Precursors from the Sp and YS were unable to produce CFU-S or long-term reconstitution of irradiated recipients. Different authors (for review, see Medvinsky et al., 1996) previously reported that CFU-S appeared in both intra- and extraembryonic locations only after the 27-somite stage (late 9.5 dpc) and long-term repopulating activity after 10 dpc (30–35 somites). Even then only 3% of recipient animals were restored. One explanation for these failures might be that earlier progenitors cannot home and settle in the adult environment in which they were transferred. The following evidence argues in favor of this interpretation. One, Toles et al. (1989) obtained long-term reconstitution by transplacental injection of YS (23% of born mice) and peripheral blood cells of 9 dpc embryos (7.5% of born mice) into 11–15 dpc W mutants. Interestingly, adult mutants were never reconstituted following injection of pre-12 dpc embryonic precursors (YS and FL), whereas HSC from 13–15 dpc FL could cure their anaemia (Harrison et al., 1979; Sonoda et al., 1983). Two, microinjection of 8–10 dpc YS cells into the YS cavity of 8–11 dpc embryonic recipients (Weissman et al., 1978) allowed the birth of chimeric animals. In the two cases where early progenitors were placed in an embryonic environment, the efficiency of reconstitution was low, perhaps due to the technical difficulty of the injection, but also to the choice of the YS or peripheral blood as donor cells.

An earlier report by Palacios and Imhof (1993) describes results conflicting with the conclusions of several other groups (Cumano et al., 1993; Medvinsky et al., 1993; Huang et al., 1994; Müller et al., 1994; Godin et al., 1995a) as well as with the results of this current study. Palacios and Imhof find that the YS but not the embryo proper displays in vitro and in vivo lymphoid potential between somite stages 3 and 6. The fact that large populations of embryonic fractions were pooled in this study might account for this discrepancy. Indeed, we found T lymphoid cell differentiation capacity in one yolk sac out of 33 independently analyzed precirculation embryos. We interpreted this result as stemming from a contamination by Sp cells as both structures constitute an anatomical continuum (see Figure 1B).

Based on the results described above, we propose the following scheme of hemopoietic ontogeny in the early mouse embryo. Hemopoietic progenitors would emerge at two different times and in two locations after gastrulation, at 6.5–7 dpc in the YS and, later, at 7.5–8 dpc in the splanchnopleura. Intraembryonic cells endowed with a high differentiation potential and maintenance abilities would mix after the onset of circulation with YS progenitors driven to fast maturation along the erythroid pathway. The Sp progenitors would persist in the P-Sp and later in the AGM, while releasing some progenitors into the circulation (Delassus and Cumano, 1996). Cells of both origins would colonize the fetal liver via the blood stream, with YS-derived cells undergoing immediate erythropoiesis, while intraembryonic precursors would give rise to definitive hemopoiesis after 12 dpc.

Experimental Procedures

Dissection of Embryonic Structures

The following mouse strains were used in the course of this study: BALB/c (Ly5.2) mice provided embryos for precursor numeration. The differentiation potential analysis was performed using embryos from the two C57BL/6 congenic lines bearing the Ly5.2 and Ly5.1 alleles of the pan-hemopoietic marker CD 45. F1 embryos resulting from the cross of the two C57BL/6 lines were also used.

Mature females were caged with breeding males. The day of vaginal plug observation was considered as day 0 postcoitum. Pregnant females between 7.5 and 8.5 dpc were sacrificed by cervical dislocation and dissections carried out as illustrated in Figure 1. Each embryo was staged by somite counting or, during presomite stages, according to the criteria of Downs and Davies (1993).

Table 2. Oligonucleotide Primers and Conditions Used for Gene Expression Analysis

Gene	Size (bp)	Annealing Temp. (°C)	5′ Sequence	3′ Sequence	Reference[a]
β major globin	578	55	5′-ctgacagatgctctcttggg-3′	5′-cacaaccccagaaacagaca-3′	1
βH1	265	55	5′-agtccccatggagtcaaaga-3′	5′-ctcaaggagacctttgctca-3′	1
ϵ globin	122	55	5′-ggagagtccattaagaacctagacaa-3′	5′-ctgtgaattcattgccgaagtgac-3′	1
mb-1	310	62 (5 cycles) 58 (35 cycles)	5′-gccaggggtctagaagc-3′	5′-tcacttggcacccagtacaa-3′	2
RAG-1	556	62 (5 cycles) 58 (35 cycles)	5′-tgcagacattctagcactctgg-3′	5′-acatctgccttcacgtcgat-3′	2
pTα	710	55	5′-taggtttgaactcagat-3′	5′-tgatttctctctgtagc-3′	3
HPRT	249	60	5′-cacaggactagaacacctgc-3′	5′-gctggtgaaaaggacctct-3′	1

[a] References: 1, Weiss et al., 1994; 2, Li et al., 1993; 3, Saint-Ruf et al., 1994.

Basic Culture Conditions

As previously described (Cumano and Paige, 1992; Cumano et al., 1992), cells were cultured on the S17 stromal line (Collins and Dorshkind, 1987; courtesy of K. Dorshkind, Riverside, CA) with the following cytokines: interleukin 7 (IL-7) used at 50–100 U/ml and interleukin 3 (IL-3) at 1:250 dilution were provided by the supernatant of transfected cell lines (from Fritz Melchers, Basel); interleukin 11 (IL-11), used at a 1:100 dilution, was provided by the supernatant of a stably transfected cell line (from R. Hawley, Toronto) (Kee et al., 1994); c-Kit Ligand (KL) (Genetics Institute, Boston) was used at 1:500, a dilution that allows the emergence of mast cells from adult bone marrow (data not shown). Murine recombinant GM-CSF (Genzyme) was used at 3 ng/ml. Human recombinant erythropoietin (Epo, courtesy of E. Goldwasser, Chicago, IL) was used at 4 U/ml.

Three Step Culture

As described in Figure 2, the YS, Sp, and occasionally the embryonic body, after resection of the YS and Sp, from staged embryos were individually explanted in wells of a 24-well plate containing γ-irradiated S17 stromal cells with IL-7, IL-11, and KL. During the first step, the embryo fragments were cultivated in toto for two days. Secondly, each explant was then individually dissociated by passage through a 26-gauge needle and cells were further cultivated for three days. The third culture step tested the differentiation potential: clones were divided into 3 fractions. Each fraction was transferred to culture conditions that promoted myeloid-erythroid, lymphoid B, or lymphoid T differentiation. In some experiments, cells obtained from identically staged structures were pooled.

Myeloid-Erythroid Conditions and Quantitation of Precursors

Cell samples (half of the culture content: 1 ml) were retrieved from Sp or YS cultures 7, 10, 12, and 14 days after explantation and the cultures were continued. Cells (5×10^3 or 5×10^4) from each sample were mixed with a culture medium consisting of OptiMEM, 0.8% methylcellulose (Fluka, 15mPAS) and 10% fetal calf serum, supplemented with IL-11, KL, IL-3, GM-CSF and Epo.

Colonies were scored at day 3 (CFU-E) and 7 (BFU-E and CFC-mix). Well-hemoglobinized clusters of less than 100 cells were classified as CFU-E. Large colonies of red cells (more than 300 cells) were counted as BFU-E whereas colonies containing at least two myeloid cell types and erythroid cells were classified as CFC-mix. Individual colonies retrieved from the culture were cytospun and stained with the May-Grünwald-Giemsa technique to confirm classification.

B-Lymphoid Conditions

Cells were cultured on S17 stromal cells with medium supplemented only with IL-7, designated here as B-cell culture conditions.

For analysis of mature B cells, cells grown in the B-cell culture conditions were stimulated with LPS (LPS: Salmonella typhosa W0901; Difco) as previously described (Cumano et al., 1993) and Ig secretion was detected in an ELISA (enzyme-linked immunosorbent assay) test.

T-Lymphoid Conditions

Cells were placed in FTOCs (Jenkinson et al., 1992) using depleted recipient thymic lobes from 14–15 dpc Ly5 congenic C57BL/6 mice, as previously described (Godin et al., 1995a). In brief, 30 μl of the cell suspension was distributed between 3–4 irradiated fetal thymic lobes in wells of a Terasaki plate and cultivated in a hanging drop for 24 to 48 hr. Thymic lobes colonized by the cells were cultured for a further 10–13 days on polycarbonate filters (0.8 μm; Millipore) floating on top of the culture medium (OptiMEM supplemented with 10% fetal calf serum). To analyze cells from repopulated thymic lobes, single cell suspensions were made by teasing the organs with two needles. The cells obtained from 3–4 thymic lobes repopulated with cells from the same embryo were pooled for FACS analysis. Sp repopulated lobes consistently yielded 1–5 $\times 10^5$ cells per lobe whereas from YS repopulated lobes yielded 5×10^3 or fewer cells.

Gene Expression Study

RNA Isolation and cDNA Preparation from Cultivated Cells

Total RNA was isolated from the cells grown from YS and Sp with TRIzol (GIBCO), according to the manufacturer's protocol. RNA extracted from adult spleen and thymus was used as positive control. Oligo(dT)-primed cDNA was prepared from total RNA derived from 5×10^5 to 2×10^7 cells using avian myeloblastosis virus reverse transcriptase (RT) in a reaction volume of 20 μl under conditions recommended by the manufacturer (GIBCO–BRL).

Semiquantitative RT-Mediated PCR

To compensate for variable RNA and cDNA yields, the amount of cDNA synthesized was calibrated using the relative expression level of hypoxanthine phosphoribosyltranferase (HPRT) as a standard. PCR was performed with primers specific for β major, βH1 and ϵ globins, and for RAG-1, mb-1, pTα, and HPRT (cf. Table 1), in 50 μl samples using 1 U Taq polymerase. This procedure was carried out in a thermal cycler (Perkin-Elmer) operating on a regimen of 94°C for 30 s, 55°C–60°C for 30 s (see Table 2 for exact temperature used) and 72°C for 30 s, for 30 cycles for all samples except for β major globin (26 cycles) and pTα (40 cycles). Ten μl of the resulting amplified material was gel-electrophoresed in Tris-borate-EDTA buffer with 1.8% agarose. All PCRs were within the exponential phase, allowing the comparison of band intensity between samples. Agarose gels were stained with ethidium bromide. Images were processed using the Graphic Converter software.

Reconstitution Experiments

Cells grown from Sp and YS from F1 embryos (C57BL/6 Ly5.1 × Ly5.2) were collected after the fifth day of culture. To test for the potential to generate CFU-S d8, 2.5×10^5 to 2.5×10^6 cells were injected into lethally irradiated (800–850 rad) C57BL/6 mice (Ly5.1 or Ly5.2) either in the retroorbital sinus or directly under the spleen capsule. Similar injections were performed using adult bone marrow. The AGM region was dissected from 35–45 somite embryos and injected either immediately or after culture in the same condition as YS and Sp. To test for CFU-S d11 and long-term reconstitution potential, the irradiated mice received in addition 5×10^5–10^6 bone marrow or splenic cells (CFU-S d11) bearing the same Ly5 allele as the recipient. Control mice were similarly injected with PBS.

After 8 to 11 days, the spleens of the recipient mice were examined for macroscopic colonies. When present, the colonies were counted, explanted, dissociated, and submitted to FACS analysis. As for long-term reconstitution studies, the recipient mice were sacrificed after 4 to 5 months. The bone marrow, spleen, thymus, and cells from the peritoneal cavity were collected and analyzed by FACS.

Cytofluorometric (FACS) Analysis

FACS analysis was performed in a FACScan under the Cellquest program from Becton Dickinson. The Ly5 alleles were characterized using the anti-Ly5.2 antibody purified from the supernatant of the hybridoma line 104.2 or the anti-Ly5.1 antibody purified from the supernatant of the A20.17 hybridoma line, both coupled to fluorescein. In all analyses propidium iodide was used for marking and excluding dead cells. The following antibodies were used to stain differentiated cells: anti-CD45R/B220 (clone RA3–6B2), anti-CD4 (L3T4), anti-TCR$\alpha\beta$ (clone H57–597), anti-TCR$\gamma\delta$ (clone GL3), Gr-1, all directly coupled to PE and anti-CD8 (Ly-2) coupled to FITC from Pharmingen (CA).

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